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## Review

# The Regulation of the Autophagic Network and Its Implications for Human Disease

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## Abstract

Autophagy has attracted a lot of attention in recent years. More and more proteins and signaling pathways have been discovered that somehow feed into the autophagy regulatory pathways. Regulation of autophagy is complex and condition-specific, and in several diseases, autophagic fluxes are changed. Here, we review the most well-established concepts in this field as well as the reported signaling pathways or components which steer the autophagy machinery. Furthermore, we will highlight how autophagic fluxes are changed in various diseases either as cause for or as response to deal with an altered cellular homeostasis and how modulation of autophagy might be used as potential therapy for such diseases.

Key words: Autophagy, ATGs, mTOR, UPR, FOXO1, HSF1, Heat Shock Proteins (HSP), Human diseases.

## INTRODUCTION

Autophagy, as a dynamic and fundamental process, has been reported to play roles in development, differentiation, cell repair, immunologic defense as well as protein quality control. Ensuring the diverse and proper functioning of autophagy in cells has been highlighted as an important aspect for the maintenance of cellular homeostasis. Inversely, failures in autophagic regulation have been associated with many pathological processes, such as muscle atrophy, neurodegeneration, tumor development and aging.

It has been generally accepted that the dynamic autophagic process is mainly executed by a series of proteins, encoded by the so-called AuTophagy related Genes (ATGs). Although the number of ATG(s) is still increasing, the function of most ATG(s) has been well characterized to orchestrate the dynamic process of the formation of pre-autophagosome, the maturation

of autophagosome and the fusion with lysosome. As the autophagic process can be affected either by producing autophagosomes or by degrading autophagosomes, the concept of the autophagic flux has been introduced which is generally used as an indicator of the activity of entire autophagic process.

The core machinery of autophagy, consisting of the ATG(s), is regulated by many signaling pathways such as the mTOR pathway, the insulin pathway and the ER stress response. How the signals are transferred to lead to activation of autophagy is still far from being understood and they may occur via both direct interactions with pre-existing ATGs and transcriptionally up-regulation of the ATGs (1-4).

Based on what has been explored about the autophagic network, we review here: 1) the core machinery of ATG(s), 2) the signaling pathways affecting the autophagic flux and 3) the implications of au-

tophagy in human diseases.

## TYPES OF AUTOPHAGY

Since the 1960s, autophagy has been gradually recognized as an important catabolic process. The main aim of autophagy is to recycle and reallocate cellular components to satisfy the change of energy requirement or to decrease the accumulation of toxic products. As such, autophagy is responsible for removing long-lived proteins, damaged organelles and protein aggregates. So far, three types of autophagy have been reported, respectively named macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) (see figure 1). The common feature they all share is that the cargo is eventually delivered into lysosomes for degradation. However, the way of cargo delivery is different.

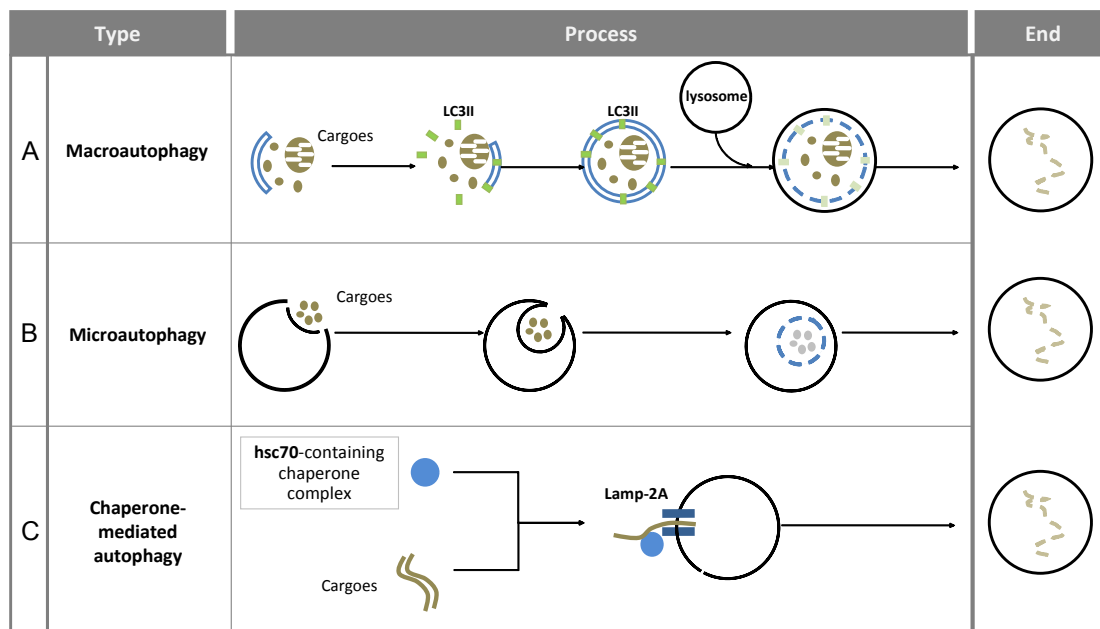
**Macroautophagy** is a multi-step and time-consuming journey for cargo wrapping and delivery. A complete process includes membrane initiation, nucleation, elongation (expansion), autophagosome maturation and the fusion with lysosome (see figure 1A). Notably, the double-membrane structure in autophagy is similar to those of other cellular plasma membranes, such as the endoplasmic reticulum, the trans-Golgi network, and mitochondria. The origin of autophagosomal membranes however is still obscure. Because of its complexity, autophagy has to be controlled precisely. On one hand, the main regulation of autophagy requires a specific group of mol-

ecules named Autophagy-related genes (ATGs). Until now, more than 30 ATGs have been discovered in yeast (5) and they are highly conserved among eukaryotes (6). On the other hand, there are many signaling pathways involved in macroautophagic induction such as the mTOR pathway, endoplasmic reticulum (ER) stress signals, the insulin pathway, and calcium signaling.

In microautophagy, the cargo is directly engulfed by the lysosome itself. Here, the targeted region of lysosomal membrane undergoes invagination, protrusion and/or septation to enclose the cargo for degradation (7) (see figure 1B). Under both basal and stress conditions, microautophagy has been reported to be involved in energy balance maintenance (8, 9).

**Chaperone-mediated autophagy (CMA)** uses KFERQ-like sequences in proteins to deliver them into the lysosome (10). The recognition of cargo and its delivery to the lysosome-associated membrane protein type-2A (LAMP-2A) is mediated by the HSPA8 (Hsc70)-containing chaperone complex (11-13) (see figure 1C).

Macroautophagy, the bulky and low-selective form of autophagy is the most investigated type, especially in relation to cellular health and pathology. Therefore, in this review we will mainly focus on the regulation of macroautophagy (hereafter generally referred to as autophagy) as well as its involvement in human diseases.



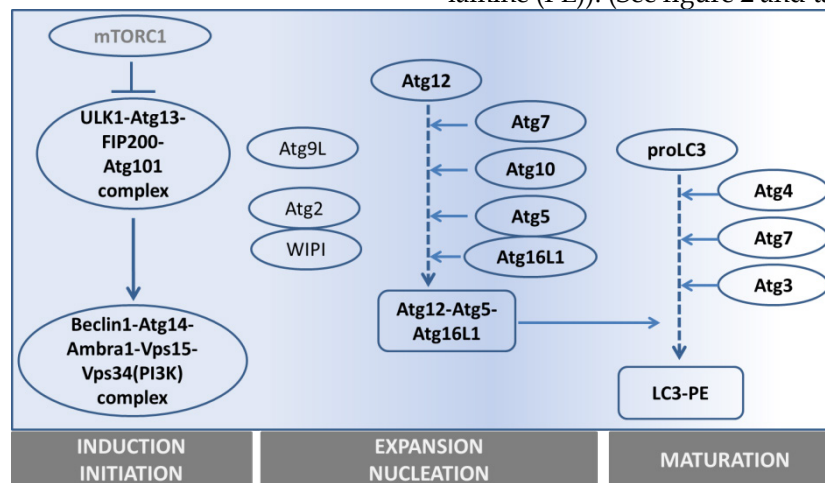
**Figure 1. Overview of the different forms of autophagy in mammals.** Macroautophagy, microautophagy and chaperone-mediated autophagy are three types of autophagy in mammals. Here, the main steps in these processes as well as the most important characteristic structures and the related mediators are presented.

## REGULATION OF MACROAUTOPHAGY

Considering the multiple roles of autophagy in both physiological and pathological processes, better understanding how autophagy is regulated in cells may enlarge our knowledge of the maintenance of homeostasis, as well as facilitate the development of efficient therapies for patients.

## Autophagosomal initiation, nucleation and maturation

The precise collaboration of ATGs is essential for performing autophagy successfully. Based on their functions, especially derived from detailed studies in yeast, ATGs have been divided into 6 groups: 1) the ATG1/ULK1 kinase complex, 2) ATG9, 3) the class III phosphatidylinositol (PI)3-kinase complex (ATG6-ATG14-Vps15-Vps34), 4) the PI(3)P-binding ATG2-ATG18 complex, 5) the ATG12 conjugation system (ATG12-ATG5-ATG16), and 6) the ATG8 conjugation system (ATG8-phosphatidylethanolamine (PE)). (See figure 2 and table 1).



**Figure 2.** Overview of the main steps in autophagy and the main genes involved.

**Table 1.** The nomenclature and functions of ATGs in yeast and mammals.

Yeast	Mammals	Functions
Atg1	ULK1, ULK2	Serine/threonine protein kinase required for vesicle formation
Atg2	ATG2A, ATG2B	Forms a complex with Atg18
Atg3	ATG3	An E2-like enzyme, which catalyzes the lipidation of Atg8-like proteins (GABARAP, GABARAPL1, GABARAPL2 or MAP1LC3A); which also autocatalyzes itself to form Atg12-Atg3 complex for maintaining mitochondrial homeostasis.
Atg4	ATG4A, B, C, D	Cysteine protease: Atg8 processing and activation.
Atg5	ATG5	Forms the Atg12-Atg5 complex, which plays E3-like activity for Atg8 lipidation
Atg6	Beclin-1	Forms the Vps34 PI3K complex
Atg7	ATG7	An E1 enzyme that mediates Atg8 lipidation and Atg12-Atg5/Atg3 interaction
Atg8	LC3/MAP1LC3A, GATE-16/GABARAP	Modifier, involves in formation of autophagosomal vacuoles through conjugating to PE.
Atg9	ATG9A, ATG9B	Cycles between a juxta-nuclear trans-Golgi network compartment and late endosomes, and interacts with the Atg2-Atg18 complex.
Atg10	ATG10	An E2-like enzyme that catalyzes the conjugation of Atg12 to Atg5.
Atg12	ATG12	Conjugated to Atg3 and Atg5
Atg13	ATG13	Downstream target of mTOR signaling pathway: the Atg1-Atg13-Atg17-Atg101 complex
Atg14	ATG14	Component of Vps34 PI3K complex
Atg16	ATG16L	Forms the Atg16L-Atg12-Atg5 complex
Atg17	RB1CC1/FIP200	Component of Atg1/ULK1 complex: Atg1-Atg13-Atg17-Atg29-Atg31 in yeast, and ULK1/2-Atg13-FIP200-Atg101 in mammals
Atg18	WIPI-1,2,3,4	Forms the complex with Atg2.
Atg29	?	Component of Atg1 complex
Atg101	ATG101	Component of the ULK1/2 complex

The ATGs uncovered so far as well as ATG functions are listed. Abbreviations: GABARAP is GABA(A) receptor-associated protein; MAP1LC3A/MAP1LC3B is microtubule-associated proteins 1A/1B light chain 3. ATG16L: autophagy related ATG 16-like; RB1CC1 is RB1-inducible coiled-coil 1. FIP200 is 200 kDa FAK family kinase-interacting protein. WIPI-1,2,3,4 is WD repeat domain, phosphoinositide interacting 1.

1) Autophagy is initiated by forming a lipid bilayer, which is either newly synthesized or budded from existing membranes in the cytoplasm (14). In *Saccharomyces cerevisiae*, the initiation site is defined as the pre-autophagosomal structure (PAS) (15). The serine/threonine-protein kinase ATG1 containing complex, supported by ATG13-FIP200-ATG101 (16-18), works as the most upstream component of the core autophagy machinery, especially after starvation (19). Unc-51 like kinase 1 and 2 (ULK1 and ULK2) are the mammalian homologues of ATG1. In yeast, ATG1 forms a complex with ATG13, ATG17, ATG29 and ATG31. However, the mammalian ULK complex has different components, including ULK1/2, ATG13, FIP200 and ATG101.

2) To facilitate membrane transport for the expansion step, ATG9, the autophagy-specific phosphatidylinositol 3-kinase (AS-PI3K) complex, ATG2-ATG18 and ATG12-ATG5-ATG16 are required. ATG9, the sole integral membrane protein, facilitates autophagic membrane transport (20).

3) The components of the AS-PI3K complex are different in yeast and mammals. In yeast, there are two PI3K complexes, ATG30-ATG14-Vps15-Vps34 and ATG30-Vps38-Vps15-Vps34, which respectively function in autophagy and vacuole protein sorting (21, 22). Among these components, Vps34 is the one known to have PI3K activity. Similarly, the mammalian homologue of Vps34, also known as PI3K class III (PI3KC3), is involved in both autophagy and endosomal trafficking. The mammalian AS-PI3K complexes are more elaborate than in yeast. They consist of Vps15, Vps34, ATG14, Beclin-1, UVRAG, Rubicon and probably more components (23, 24). Among them, Beclin-1 (the mammalian homologue of ATG6) is the crucial one differentiating the autophagic PI3KC3 complex from the endosomal one, and it is essential for autophagosome formation (25). Beclin-1 can either up-regulate or down-regulate autophagic activity through binding with different partners. Ambra1, Bif-1, and VMP-1 are Beclin-1 partners that activate autophagy, whereas, its partners Bcl-2, ICP34.5 and M11 inhibit autophagy (26-28).

4) The ATG2-ATG18 complex is required for nucleation. Especially its PtdIns(3)P-binding ability is critical for the complex to localize to autophagic membranes, endosomes or vacuolar membrane (29).

5) The ATG12-ATG5-ATG16 complex mediates ATG8 lipidation (see next paragraph). The activating process of ATG12 shares similarities with the ubiquitin-ligation process (consisting of an E1-ubiquitin activating enzyme and E2-ubiquitin-conjugating enzymes): ATG7, as E1-like protein, firstly catalyses ATG12 to become an active form, and next ATG10 (an E2-like protein) facilitates the active ATG12 to bind

with ATG5. ATG12-ATG5 further assembles into a 350kD complex by interacting with the small coiled-coil protein ATG16 which is essential for autophagy (30).

6) The ATG8 conjugation to phosphatidylethanolamine (PE) is crucial for autophagosomal expansion and maturation. Therefore, ATG8 or its mammalian homologue, LC3, is generally used as a marker to trace the autophagic process (see next section) (31, 32). To fulfill ATG8-PE conjugation (ATG8 lipidation), two possible routes can be used. One is via assisted assembly by the ATG12-ATG5-ATG16 complex mentioned above (33); another is via processing LC3 itself. Newly synthesized LC3 is firstly cleaved by ATG4 to become a cytosolic form (LC3 I), then a fraction of LC3I is further activated by ATG7 (an E1-like protein) (34) and ATG3 (an E2-like protein) (35) to become LC3II (the membrane-binding form). Even though the core machinery of autophagy has been rolled out as being complex already, the number of components discovered to be involved in this network still increases (36).

## MEASURING THE AUTOPHAGIC FLUX

The activity of autophagy is controlled by autophagosomal formation (input) and autophagosomal degradation (output). Therefore, the speed of the autophagosomal turnover is defined as autophagic flux, which is the real indicator of autophagic activity. How to measure the autophagic flux has been excellently described elsewhere (37).

Here, we only shortly summarize the markers and inhibitors that are most commonly used.

1) As mentioned above, LC3 lipidation is essential for autophagy to proceed. This lipidation of LC3 can be demonstrated by western blot analysis (WB) (38). Upon an increase in autophagosomes, the band of LC3 will shift from LC3I (18kDa) to LC3II (16kDa) on SDS-PAGE. Upon time after autophagic activation, levels of LC3II will decline again because it is co-degraded with the autophagic cargo in lysosomes.

Whilst LC3 data are indicative of a changed autophagic flux, they cannot distinguish whether more autophagy is induced or whether autophagosomal-lysosomal fusion is impaired, unless accurate kinetics are studied. To distinguish between these possibilities, one can block either the synthesis or degradation of LC3II using autophagic inhibitors (see figure 3). Wortmannin and LY294002 can inhibit the autophagosome formation: if LC3 is still lipidated by the experimental condition in the presence of these inhibitors, this implies that autophagosomal-lysosomal fusion was impaired. Bafilomycin A1 and the inhibitors of lysosomal proteases (such as E64,



pepstatin A, chloroquine) stop LC3II degradation, This in itself will further increase LC3II levels; if under the experimental condition LC3II levels even further increase, this then implies that the condition has led to an increased autophagosome formation.

2) p62/SQSTM1 is thought to be responsible for cargo selecting and transport of proteins or protein aggregates (not organelles), and is also co-degraded with its cargo in lysosomes (39). Thus, p62 degradation can also be used to measure the autophagic flux, which should then be delayed by bafilomycin A1 and the inhibitors of lysosome proteases.

Besides measuring LC3II and p62 biochemically, one can also use microscopic analyses as they form characteristic puncta. The number of these puncta can be counted as (semi) quantitative analyses of autophagy.

3) Still the best way to analyze the induction of autophagy and to demonstrate that the observed puncta/vesicles are indeed autophagosomes is by using Electron Microscopy(EM) in which it can be recognized as a vesicle surrounded by a double membrane that contains cytosol, protein aggregates, and/or organelles that look morphologically intact.

## AUTOPHAGY REGULATING SIGNALING PATHWAYS

Autophagy is regulated by many signaling pathways. The most established ones of these are discussed below.

### The mTOR pathway

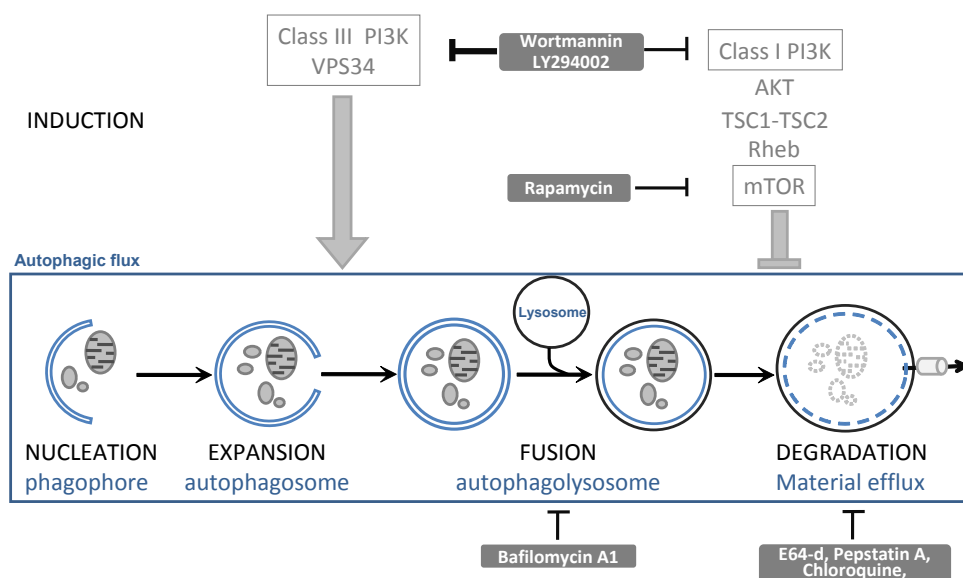
The mTOR (mammalian target of rapamycin) serine/threonine kinase is a well-characterized and critical node of cell metabolism (40) and (besides controlling autophagy) mainly regulates ribosomal bio-

genesis and protein translation (41). The mTOR pathway is the most extensively studied network for autophagy regulation because of its ability to timely sense the nutrient state, growth factors availability and stress (42). It is still unclear how mTOR itself is regulated, although the phosphatidylinositol 3-kinase (PI3K)-AKT/protein kinase B (PKB) (43), TSC1/TSC2 (44, 45), Rheb (Ras homolog enriched in brain) (43), PRAS40 (46) and DEPTOR (47) have been found as its regulators under different conditions.

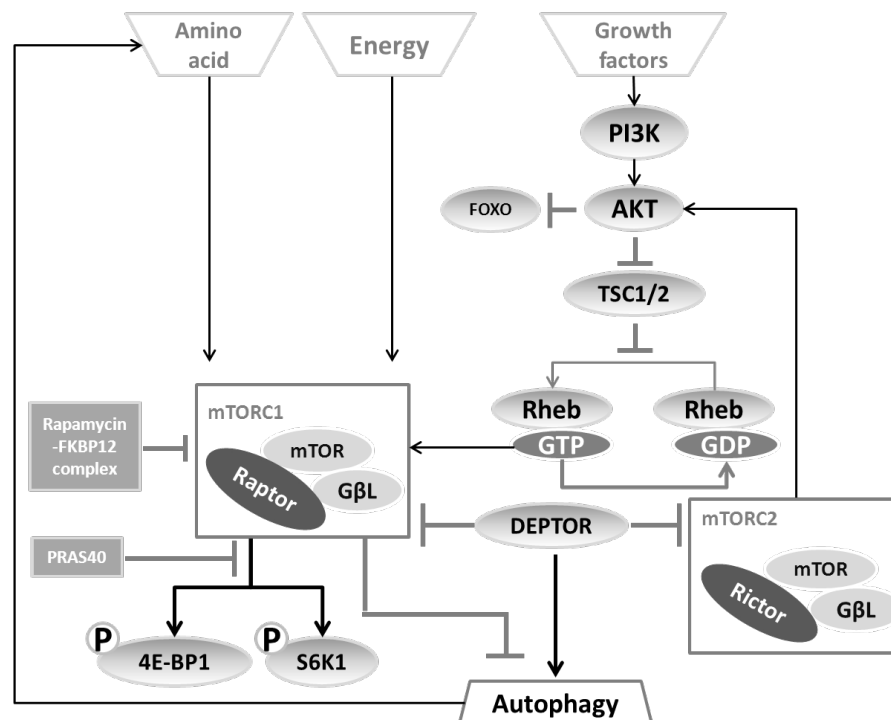
So far, two distinct mTOR complexes have been found in mammals (see figure 4).

(i) The first complex, mTORC1, includes mTOR, the mammalian LST8/G-protein  $\beta$ -subunit like protein (mLST8/G $\beta$ L), and the regulatory-associated protein of mTOR (Raptor). Based on the observation that the FKBP12-rapamycin complex can destabilize the mTOR-G $\beta$ L-Raptor complex (41), rapamycin has been generally used as a mTORC1 inhibitor leading to autophagy induction (48, 49). As substrates of mTORC1, S6K1 (50) and 4E-BP1 (51) can be modulated to change the recruitment of ribosomal subunits as well as the efficiency of cellular translation, thereby balance the protein synthesis to fit with cellular requirement for energy. For this reason, the phosphorylated state of S6K1 and 4E-BP has been considered as a convenient indicator of mTORC1 activation (52).

(ii) The second complex, mTORC2 consists of mLST8/G $\beta$ L and Rictor (rapamycin-insensitive companion of mTOR). This branch regulates the Akt-FOXO axis and the PKC $\alpha$  pathway, but not S6K1 or 4EBP1 (53). mTORC2 has been considered as rapamycin-insensitive complex (54). Nevertheless upon prolonged treatment, rapamycin also impedes on mTORC2 (55).



**Figure 3. The autophagic flux and generally used inhibitors.** Inside the square, the autophagic flux is depicted. This is a dynamic process that can be modulated by the indicated drugs (grey text boxes) by affecting autophagosome induction and expansion (ON-rate) or by affecting autophagosome fusion or degradation (OFF-rate).



**Figure 4. Regulation of the two mTOR complexes.** mTORC1 is able to sense the amino acid concentration and energy state directly, but needs the PI3K-AKT axis to sense growth factor status. The downstream effects of mTORC1 are mainly mediated by 4E-BP1 and S6K1, which can be inhibited by the rapamycin-FKBP12 complex. mTORC2, as a brake-like inhibitor, decreases the extent of AKT-induced mTORC1 activation.

## Calcium signaling pathway

Calcium signaling is another generally studied, but still obscure pathway by which autophagy can be regulated. In cells, a stable calcium concentration is maintained by  $\text{Ca}^{2+}$  channels through two independent but closely linked processes (56): 1) a slow, sustained entry of extracellular calcium, and 2) a rapid, transient release of calcium from stores in the ER. To replenish or maintain  $\text{Ca}^{2+}$  stores, Voltage-Operated Channels (VOCs), Receptor-Operated channels (ROCs) and Store-Operated Channels (SOCs) are required (56, 57). Importantly,  $\text{Ca}^{2+}$  release is only mediated by inositol 1,4,5-trisphosphate ( $\text{InsP}_3/\text{IP}_3$ ) receptors ( $\text{IP}_3\text{Rs}$ ) in the ER that thus play a critical role in transient  $\text{Ca}^{2+}$  signaling (56). Not surprisingly,  $\text{IP}_3\text{R}$  and TRPML3 (a member of the TRPML subfamily of the transient receptor potential (TRP) channel superfamily) have been shown to respectively inhibit and promote autophagy (58, 59). In line with this, it has been also shown that the expanding ER is a potent stimulator of autophagy (60).

Importantly, the concentration of calcium directly links with the activity of calcium-sensitive kinases and proteases. On one hand, Calmodulin-Dependent Kinase Kinase- $\beta$  ( $\text{CaMKK}\beta$ ) (61), elongation factor-2 kinase (eEF-2 kinase;  $\text{Ca}^{2+}$ /calmodulin-dependent kinase III) (62) and AMP-activated protein kinase (AMPK) (63) will be

activated by abnormal cytoplasmic concentrations of calcium under stressed conditions. Each of these upstream sensors will immediately transmit the signals to downstream substrates for adjusting cellular metabolism in new microenvironment and autophagy will be activated to release more amino acids under these conditions. On the other hand, higher  $\text{Ca}^{2+}$  concentration increases activity of calpain which can cleave ATG5, thereby inhibits autophagosome formation (64).

## The Unfolded Protein Response

Except being an important regulator of cytosolic calcium concentration, the ER is the main cellular protein factory that synthesizes membrane bound and secretory proteins (65). Protein homeostasis in the ER is controlled by the Unfolded Protein Response (UPR) that consists of three parallel branches: activating transcription factor 6 (ATF6), double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK), and inositol requiring enzyme 1 (IRE1). These branches serve to not only increase the folding capability of the ER (through induction of chaperones), but also enhance the degradation capability. This involves both ER-associated proteasomal degradation (ERAD) as well as autophagy (see figure 5) (66).

(i) IRE1 (an endoribonuclease, presents in the ER membrane) is the most conserved branch of the UPR (67). Once activated, IRE1 cleaves the mRNA of XBP1

(X-box binding protein 1). This step produces XBP1<sup>s</sup> (the spliced mRNA) which transcriptionally up-regulates UPR related genes. Generally, IRE1 is responsible as the driving force of the entire UPR gene program (68). XBP1 also up-regulates the BAG3/HSPB8 complex (69), which recently has been found to be involved in the induction of autophagy (70).

(ii) ER located ATF6 can be cleaved by site-1 and site-2 protease (S1P and S2P) to yield a cytosolic fragment (ATF6c) that can translocate into the nucleus for up-regulating UPR genes, such as BiP (the ER-resident chaperone of the heat shock protein HSP70 family), protein disulfide isomerase (PDI) and GRP94 (glucose-regulated protein 94, the ER-resident chaperone of the Hsp90 family) (71). Whilst the ATF6 pathway primarily serves to induce the expression of ER chaperones, ATF6 can also up-regulate the autophagy-inducing BAG3/HSPB8 complex under certain conditions (Minoia et al., unpublished), indicating that also this UPR-branch can increase the autophagic flux.

(iii) The 3<sup>rd</sup> UPR branch, PERK, has as main function to decrease the burden of newly synthesized ER proteins. PERK phosphorylates and inactivates translation initiation factor 2 $\alpha$  (eIF2 $\alpha$ ), which causes a general block in mRNA translation. Meanwhile, ATF4 is specially induced by eIF2 $\alpha$  through recognizing its 5'-untranslated region, allowing the selective up-regulation of transcription factor C/EBP homologous protein (CHOP) and the activation of growth arrest and DNA damage-inducible 34 (GADD34). GADD34 then plays as a negative feedback loop for the response essential for cell survival (72, 73). Interestingly, however, ATF4 is also involved in autophagy activation as it leads to the induction of ATG12 (74).

### Insulin (IS) pathway

As one of the most important signaling pathways in cell metabolism, the Insulin/IGF (insulin-like growth factors) axis is responsible for promotion of cell proliferation and inhibition of cell death, which are mediated mainly by the PI3K-AKT/PKB pathway and the Ras-mitogen-activated protein kinase (MAPK) pathway (75-77). The Insulin/IGF axis has many substrates, two of which being the forkhead box O class transcription factors (FOXOs) and the heat shock transcription factor 1 (HSF1) (78). Whilst the FOXO pathway has in various ways been linked up with protein degradation systems, especially autophagy (1, 79), the HSF1 branch of this network has been mainly thought to be involved in enhancing the protein folding capability of cells (80). Yet, also HSF1 activity can lead to enhancement in autophagic activ-

ity (see below).

Most of the pioneering studies on FOXO and HSF1 were done in *C. elegans*. Both DAF16 (FOXO homologue in *C. elegans*) and HSF1 were found to be crucial for the downstream effects of the insulin pathway on life span and the ability to handle diseases associated (misfolded) proteins (81-84). Below, we will shortly summarize those data.

### (a) FOXO

While there is only one DAF-16 in *C. elegans* and one dFOXO in *flies*, the mammalian FOXO family includes 4 members, FOXO1/FKHR, FOXO3/FKHRL1, FOXO4/AFX, and FOXO6 (85). FOXO6, expressed in a specific temporal and spatial pattern, is believed to be mainly involving in brain development (86). Currently, FOXO1, FOXO3 and FOXO4 are known to share the most elementary and conserved sequence motifs as well as modification features (87): 1) the DNA-binding domain (or forkhead (FKH) domain), which recognizes (G/C)(T/C)AAA(C/T)AA or TT(G/A)TTT(G/A)(G/C) in the promoter of target genes in mice; 2) the three AKT1-phosphorylation sites (Thr24, Ser256, and Ser319 in human FOXO1) that mediate nuclear-cytoplasm shuttling (88, 89). Generally, the members of the family can compensate for each other (90). However, in knock-out mice different FOXOs do show some specific functions and expression patterns: FOXO1 in vascular development, FOXO3 in ovarian follicular development, and FOXO4 without any distinguishable phenotype (91).

Nuclear (active)-Cytoplasm (inactive) shuttling is a critical and generally accepted model for regulating the transcriptional activity of FOXOs. Except for the PI3K-AKT/PKB pathway that mediates the nuclear export of FOXO, many other upstream modulators do in parallel affect the location of FOXOs, including various kinases and phosphatases (SGK, CK1, AMPK, JNK, MST1, IKK $\beta$ , DYRK1, CDK2, and PP2A), acetylases and deacetylases (CBP/P300, PCAF, SIRT1/2) and ubiquitinases and deubiquitinases (USP7/HAUSP, and Skp2) (87, 92). Irrespective of how FOXOs are activated, the consequence will be a change in the expression profile of its large set of target genes that regulate many cellular processes including autophagy (93) (see table 2). In line, alterations in FOXOs activity have also been related to many pathological processes, such as diabetes, cancer, infertility, cardiovascular disorder, immune system dysfunction, neurodegeneration and ageing (94-97).

Regarding the role of FOXOs in autophagy two models of action have been described. In line with its function as transcription factor, FOXO3 can induce autophagy through transcriptionally up-regulating LC3, Gabarapl1, Vps34, ULK2 and ATG12 (99).



FOXO3 induced autophagy entirely seems to depend on FOXO1 since lack FOXO1 prevents FOXO3's function in autophagy (106). Surprisingly, however, FOXO1 (also) can boost the autophagic flux in a transcription independent manner by direct binding to ATG7 in the cytosol (1). This novel and unprecedented action of FOXO1 might explain to some extent why DAF-16 mediates such a large life span expansion and leads to pronounced protection against neurodegenerative protein aggregation diseases in *C. elegans* (107, 108) (also see below).

**Table 2.** The downstream targets of FOXOs.

Target genes (ref)	Cellular processes	Consequences
p21 <sup>Cip1</sup> , p27 <sup>Kip1</sup> (98)	Cell cycle	Oncogenesis
FasL, Bim, Bnip3 (98)	Apoptosis	
LC3, Gabarapl1, Vps34, ULK2, and ATG12 (99)	Autophagy	Longevity Suppression of neurodegeneration
Catalase, Sod2, Gadd45, DDB1 (98), caveolin-1 (100)	Stress resistance	
Sprouty2, PBX1 (90), Cited2 (101)	Angiogenesis	Differentiation
BTG1, KLF4 (102),	Differentiation	
NPY (neuropeptide Y), AgRP (Agouti-related protein) (103), Pomc (104)	Food intake	Metabolism
Atrogin-1, MuRF1 (105)	Ubiquitin-proteasome	
		Muscular Degeneration

### (b) HSF1

As another downstream target of the Insulin/IGF axis, HSF1 is essential for development and stress resistance (109). HSF1 is known as the major factor involved in the up-regulation of heat shock proteins serving to restore proteostasis in the cytosol (81, 110). Under stress conditions, HSF1 is thought to release from the Hsp90 docking complex that normally keeps it inactive under non-stress conditions, and may undergo some structural changes by not yet elucidated mechanisms. Then, HSF1 translocates into the nucleus where it gets hyperphosphorylated and binds to HSE in promoter of target genes (mainly HSPs). Next, the acetylation of HSF1 seems required for its release from DNA and the turn-off of gene expression (111).

In mammalian cells, HSPs are divided into HSPC (HSP90), HSPA (HSP70), DNAJ (HSP40), HSPB (small HSP) and chaperonins (CCT, GroES/EL) (112). Whilst HSF1 regulated HSPs generally seem to serve in either protein folding or proteasomal degradation, recent

evidence has suggested that some of its target HSP genes are also linked to autophagy. E.g. Carra *et al* demonstrated that HSPB8 in complex with BAG3 and Hsp70 can increase the autophagic flux and thereby enhance the clearance of protein aggregates which arise during neurodegenerative diseases (70, 113) (see figure 6). Except as UPR target genes (see figure 5), HSPB8 and BAG3 can also be induced by activation of HSF1 (114). Another small HSP, HSPB7, also serves in autophagic clearance of model aggregation putatively through improving loading of cargo (the aggregates) into autophagosome (115). How HSPB7 is regulated, however, is still unknown.

## THE ROLE OF AUTOPHAGY IN NORMAL PHYSIOLOGY AND DISEASE

### Physiological roles of autophagy

Autophagy has mostly been studied as a mean of cells to cope with acute or chronic stresses, where metabolism needs to be adjusted rapidly to fit with the changed microenvironments (116-118). However, autophagy is also indispensable under normal-growth conditions (64, 119) and the physiological roles of autophagy include:

#### (a) Nutrients recycling

Through degradation of cellular materials, autophagy facilitates an efficient and dynamic energy conversion of amino acids, glucose, lipids and nuclear acid, especially under conditions of starvation (118, 120, 121).

#### (b) Cell self-Repair

Autophagy is required for removal of damaged mitochondria (mitophagy), peroxisomes (pexophagy) and protein aggregates (aggrephagy) as arise during normal cell growth (39, 122).

#### (c) Protein quality control

Protein quality control is mastered accurately by balancing protein synthesis, protein folding and protein degradation. Unlike steric considerations for proteasomal degradation, autophagy shows more general or non-selective property. Autophagic and proteasomal degradation are closely linked with each other, which is exemplified by a boost of autophagy after proteasome inhibition (123, 124).

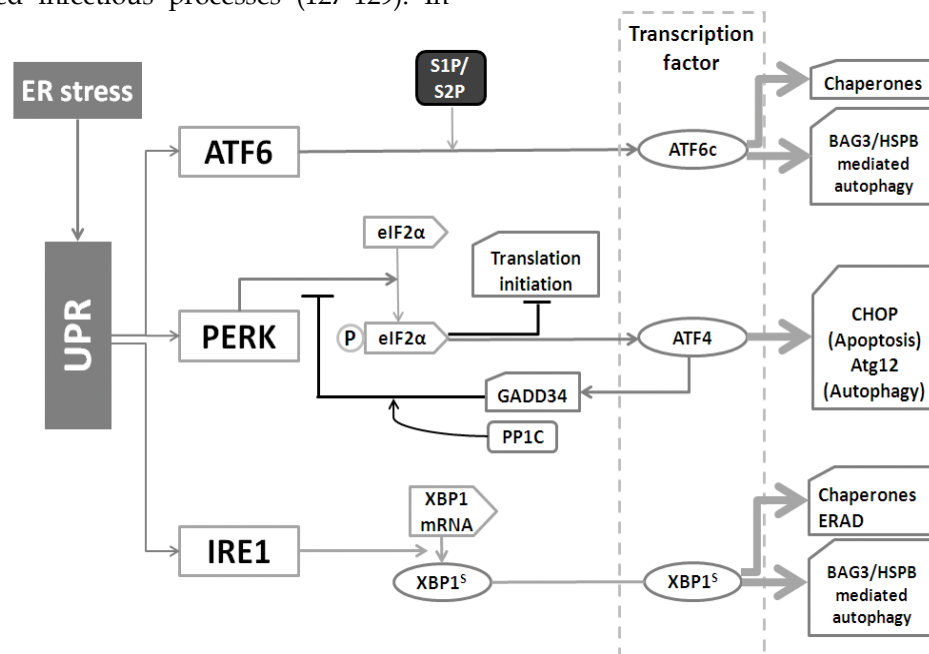
#### (d) Cell maturation and differentiation

As evidenced from the phenotype in ATG knock-out mice, autophagy also affects differentiation (125). Similarly, in *Drosophila*, autophagy is essential for remodeling of the larval mid-gut as well as the development of the larval salivary gland (126).

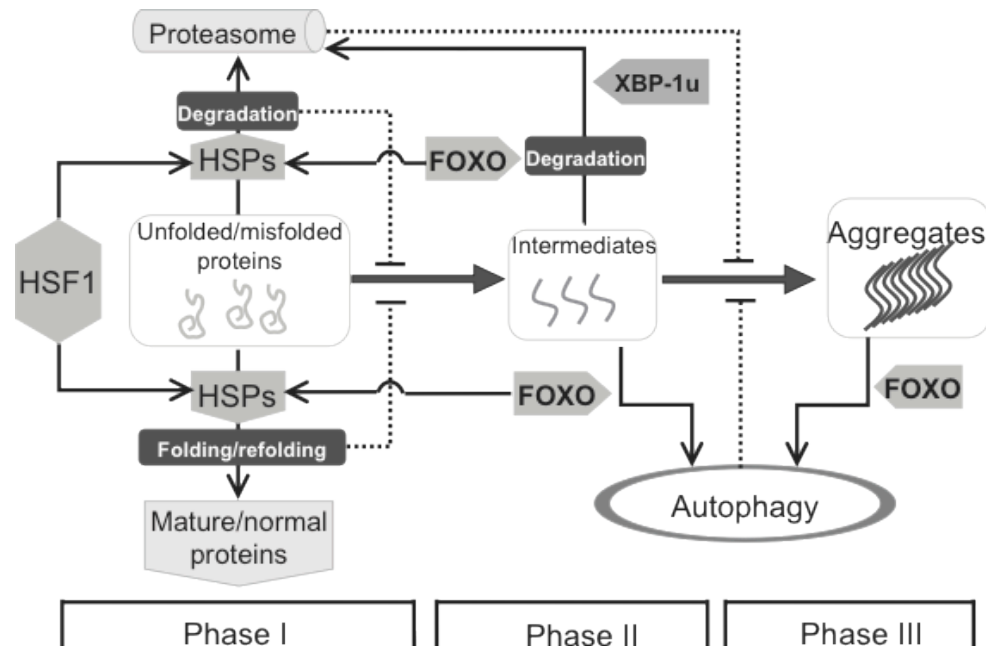
## (e) Infection and immunity

Autophagy is involved in both bacteria-related and virus-related infectious processes (127-129). In

addition, Beclin1/Atg6 mediated autophagy impedes on lymphocyte development (130).



**Figure 5. The different branches of the Unfolded Protein Response (UPR) and autophagy.** The UPR is mediated by the ATF6, PERK, and IRE1 pathways. 1) After cleavage, ATF6 is able to up-regulate the expression of chaperones. 2) PERK is responsible for eIF2 $\alpha$  activation. Activated, phosphorylated eIF2 $\alpha$ -p then inhibits translation initiation, meanwhile stimulates ATF4 to transcriptionally activate CHOP and Atg12. In addition, ATF4 also activates GADD34 to decrease the activity of eIF2 $\alpha$ -p, which forms a feedback loop. 3) IRE1 cleaves XBP1 to increase the expression of chaperones as well as speed up ERAD.



**Figure 6. Protein homeostasis is mainly maintained by HSF1 and FOXO, two downstream targets of the insulin pathway.** The formation of biochemistry-detectable aggregates is a dynamic process, which is initiated by accumulation of unfolded/misfolded proteins (Phase I), facilitated by the formation of intermediates (Phase II) leading to the appearance of detectable aggregates (Phase III). This process can be intervened by different cellular strategies, including HSPs mediated folding/refolding, as well as proteasome- and autophagy-mediated degradation. HSPs, up-regulated by HSF1 and FOXO, are mainly responsible for Phase I, in which the misfolded proteins can be either refolded to become functional normal proteins or degraded if refolding is unsuccessful. Once the intermediates are formed, autophagy starts playing a more important role. At the beginning of Phase II, the intermediates can still be handled by either HSPs or proteasome, and this response supposedly requires FOXO. At both Phase II and III, FOXO is also involved the induction of autophagy to clear large(r) protein aggregates.

#### (f) Cell death

Controlling cell fate properly is important for development, differentiation and tumor suppression. Autophagy initially provides the adaptive response to micro-environmental change or stress. However, if the stress persists or the amount of inflicted damage goes beyond of cellular tolerance, exaggerated autophagy will lead to cell death and thus remove the damaged cells to maintain tissue and organismal integrity. As such, autophagy parallels apoptosis which also primarily serves as a cell remodeling process under stresses, but if over-activated helps cell elimination (131).

### Autophagy and diseases

Deregulation of autophagy has been linked with loss of cell function leading to organismal pathologies (132, 133). In many cases, it is not clear whether deregulation of autophagy itself is the trigger for such pathologies or just a mere consequence and reflection of alterations in the amount of cargo for the autophagic degradation. Below, a few links between altered autophagy and diseases will be summarized.

#### (a) Tumor development

Tumorigenesis is a multi-step process, involving oncogenes and tumor suppressor genes. Several of these genes, e.g. p53, FOXOs, TOR, SirT1/2, Bcl-2, PTEN, Ras, NF- $\kappa$ B and series of micro RNAs, also have effects on autophagy (134). On one hand, autophagy could directly play a role in tumor suppression. Firstly, mutations in or deletion of Beclin-1, which is the essential ATG for autophagy initiation are found in 40–75% of cases with human breast, ovarian or prostate cancer (25). Even more so, deficiency of Beclin-1 enhances tumorigenesis of lymphomas, lung carcinomas, hepatocellular carcinomas and mammary precancerous lesions in mice (135). Secondly, FOXO1, except as transcription factor to induce the expression of tumor suppression genes, alternatively can bind to ATG7 to activate the autophagic cell death, hereby antagonizing tumor development (1). Why and how this FOXO1 related function is silenced during tumorigenesis is yet unclear. However, it was recently found by Zhao et al (136) that the unspliced form of XBP-1(XBP-1u) can promote ubiquitin-independent proteasomal degradation of FOXO1 and may thus be considered as an oncogene stimulating tumor initiation.

On the other hand, autophagy can be up-regulated to support tumor development, e.g. under hypoxic conditions, tumor cells require autophagy to cope with the low nutrient availability (137). In addition, autophagy may provide tumor cells with resistance to chemotherapy (138).

So, it seems that impaired autophagy can both enhance and lower the risk of tumorigenesis (139). Which of these effects prevail likely is dependent on the stage of tumor, tumor physiology and the tissue from which the tumor originated.

#### (b) Liver diseases

The liver, the most important tissue for organismal metabolism, is responsible for: 1) amino acid metabolism (synthesis and degradation), 2) glucose metabolism (gluconeogenesis, glycogenolysis and glycogenesis), 3) lipid metabolism (cholesterol synthesis, lipogenesis and lipoproteins synthesis), 4) blood cell regeneration, blood ingredient replenishment and coagulation reaction, 5) endocrine system homeostasis (secretion of IGF-1, breakdown of insulin and other hormones), 6) detoxification (endogenous toxic metabolic products and exogenous medicine) and 7) completion of immunological effects. Not surprisingly, autophagy is a critical process in hepatocytes, and abnormal autophagy has been observed in many pathological liver-related disorders, such as insulin resistance, nonalcoholic steatohepatitis (NASH), cirrhosis, viral hepatitis, hepatic steatosis, mutant  $\alpha$ 1-Antitrypsin Z protein related diseases and liver cancer (140). What kind of role autophagy is playing in these diverse liver pathologies is still largely unknown. Recently, we revealed that in polycystic liver disease (PCLD), autophagy is activated by the deficiency of hepatocystin, of which mutants have been shown to cause PCLD (141). Therefore, in PCLD, autophagy inhibition might be a strategy to delay pathogenesis.

#### (c) Brain and neurodegenerative diseases

Autophagy has been shown as an essential process for the maintenance of neuronal function (124). This theory is supported by the observation that in neurodegeneration such as Alzheimer's, Parkinson's, Huntington's disease and amyotrophic lateral sclerosis (ALS) protein aggregates have accumulated indicative of disturbed proteostasis, including impaired autophagy. Indeed, several lines of evidence support that boosting autophagy in such diseases may have beneficial effects (142–144). However, over-stimulating autophagy has the risk to become cytotoxic and pro-degenerative. Therefore, besides increasing the autophagic flux, improving cargo (protein aggregate) delivery into autophagosomes seems a promising strategy to counteract neurodegeneration. This might be accomplished by e.g. stimulating proteins like Alf1 (145), or boosting certain HSP like HSPB7 (115, 146).

## CONCLUSION AND PERSPECTIVES

Autophagy is not merely a strategy of cellular catabolism to recycle proteins and other nutrients, but also responsible for many other cellular functions, such as cellular quality control which requires proper clearance of misfolded proteins, damaged organelles, and sometimes ensuring clearance of damaged cells. Although the regulation of the autophagic network has been comprehensively investigated in the recent decades, it is still controversial whether a dysfunctional autophagy is actually the cause for a disease or just an indicator of upstream protective response that has failed to protect cells and tissues from an injury. Nevertheless, boosting the autophagic flux has shown the significant benefits in various disease models (116, 119, 132, 143), implying that its activity at least does influence progression of certain diseases. How to modulate the autophagic network precisely to do so without impeding on normal cellular physiology, i.e. avoiding toxicity, will however remain a major challenge. Precise knowledge on the many different signaling pathways that are involved in the regulation of the autophagic network will therefore be crucial and require co-induction of upstream protective pathways such that damage clearance is also accompanied by preventing further damage. As such, the coordinated inhibition of the insulin pathways probably sets the best example. Here, longevity and stress resistance seems is not associated with induction of autophagy, but also with an increase in molecular chaperones, and improved proteolytic activity (figure 6) (147, 148).

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## Competing Interests

The authors have declared that no competing interest exists.

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